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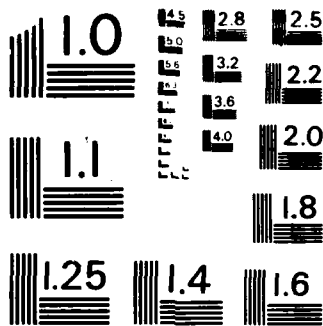   RSRE-MEMO-3977 DRIC-BR-101028

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CONTROL OF HEPATITIS VIRUS INFECTIONS BY NEW METHODS

ANNUAL AND FINAL REPORT

by

Joseph L. Melnick, Ph.D.

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Studies were performed in a young adult population with an HBsAg/adw polypeptide vaccine prepared from native intact HBsAg particles. It was demonstrated that on a weight basis the polypeptide vaccine was superior to Heptavax-B in generating an anti-HBs response, indicating that the critical antigenic determinants for inducing an anti-HBs response are associated with the low-molecular-weight polypeptides of HBsAg. In addition to the excellent peak antibody response generated by the polypeptide vaccine,

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20. Abstract (cont.)

the production of anti-HBs in vaccinated individuals was very rapid after the initial immunization. This observation has ramifications for postexposure prophylactic vaccination and indicates that the polypeptide vaccine would be a good candidate for such usage.

We have continued our investigations with anti-idiotype reagents that recognize a common idiotypic determinant on human anti-HBs molecules. Several important immunochemical parameters were established: (i) Injection of mice with anti-idiotype antibodies prior to HBsAg inoculation enhanced the anti-HBs response. (ii) Primarily IgM anti-HBs was produced if the anti-idiotype antibodies were administered in a soluble form. (iii) The anti-HBs response was increased when anti-idiotype antibodies were given in conjugation with a cyclic synthetic HBsAg peptide. This anti-HBs response was comparable to a single injection of HBsAg. Additional

It also was demonstrated that anti-HBs could be induced by injecting anti-idiotype antibodies alone. The anti-idiotype-induced anti-HBs expressed an interspecies idiotype that is shared by human anti-HBs produced by natural infection with HBV. In addition, the anti-HBs recognized the group-specific a determinant of HBsAg. These data suggest the potential use of anti-idiotypes as immunopotentiators of the anti-HBs response.

A case of posttransfusion hepatitis type A infection was documented. A young girl manifested icteric hepatitis A 31 days after receiving a unit of packed erythrocytes from a donor who subsequently died. Hepatitis A virus (HAV) antigen was detected in the donor's hepatocytes and in plasma obtained from the original donor unit. Infectivity of the donor plasma was confirmed when HAV was transmitted to a chimpanzee.

A gentle procedure was used to subfractionate microsomal compartments in an attempt to localize HAV antigen to specific sites within the hepatocyte. Most of the antigenic activity was evenly divided between the cytosol fraction and the microsomal suspension. These data provided additional evidence that replication of HAV occurs primarily within the cytoplasm of the host cell, as observed with other enteroviruses.

Foreword:

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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A. Plasma-Derived Polypeptide Vaccine (HBsAg/adw) in a Young Adult Population

1. Overview

The plasma-derived HBsAg/adw polypeptide vaccine used in this study was composed of the major gene product of HBV. The nonglycosylated form has a molecular weight of 25,000 daltons, whereas the glycosylated form has a molecular weight of 30,000 daltons. These polypeptides were packaged in a micellar configuration, alum-adsorbed at a concentration of 0.02 mg alum/µg protein, and suspended in 0.01 M phosphate buffer, pH 6.3. Thimerosal was added to the finished product at a final concentration of 1:20,000. The vaccine was administered in the deltoid region by the intramuscular route at a concentration ranging from 1.6 to 40 µg/ml.

The polypeptide vaccine was prepared from native intact HBsAg particles supplied by Dr. John Gerin, Division of Molecular Virology and Immunology, Georgetown University, Rockville, Md. The native intact HBsAg particles had been evaluated by our laboratory under a National Institutes of Health Investigational New Drug permit from 1979 through 1982. Prior to use as a vaccine product, the bulk material obtained from Dr. Gerin had undergone safety and sterility testing in experimental animals, including chimpanzees, and in humans. It was found to be free of infectious hepatitis B virions or other contaminants. In addition, the product was formalin-inactivated. This bulk material served as the starting material for our HBsAg/adw polypeptide vaccine. A description of its preparation is given below. All preparative steps were carried out in a Baker Sterigard hood that had been certified as meeting NIH 03 112-C requirements for a class II, type B hood. Glassware and equipment (columns, fraction collectors, etc.) were sterilized by steam autoclaving or ethylene oxide gas by The Methodist Hospital Central Supply. All buffers were filter sterilized using sterile 0.22-µm Millipore filter units. Personnel wore sterile gloves and were masked and gowned when preparing this product. Sterile drapes covered the working surface. The hood was wiped with 0.5% sodium hypochlorite daily.

All buffers and the final product were tested for endotoxins by Mr. Norman Peterson of the Centers for Disease Control Laboratories in Phoenix, Ariz., using a Limulus lysate assay; for mycoplasma by The Methodist Hospital Virus Laboratory (21-day incubation at 37°C on PPLO agar in an anaerobic environment); and for aerobes, anaerobes, and fungi by the Special Infectious Diseases Laboratory at The Ben Taub General Hospital (incubation on chocolate agar for 72 hr, incubation in an anaerobic blood tube for 72 hr, and incubation in Sabouraud's agar for 4 weeks).

After thimerosal was added to the bulk material and the 40 and 8 µg/ml dosages were vialed, two terminal vials from each concentration were tested for anaerobes, aerobes, and fungi; two vials were tested for mycoplasma; and two vials were tested for endotoxins as described above. In addition, the contents of one vial were put through a Millipore filter, and the filter was incubated. All samples were negative for mycoplasma, aerobes, and fungi before and after thimerosal was added. Endotoxin was detected in the batch preparation at a level of 0.1 ng/ml and in the 40 µg/ml concentration at levels of 0.05 and 0.026 ng/ml. Endotoxin was not found in the 8 µg/ml vaccine vials. According to the Centers for Disease Control (Mr. Peterson), at least 70 ng/ml endotoxin is needed to elicit a pyrogenic effect in an average-sized man. This means that at least 700 ml of vaccine would need to be injected to produce a pyrogenic response.

Alum from each of two vials of the HBsAg/adw polypeptide vaccine containing 40 and 8 µg/ml protein was solubilized in sodium citrate by dialysis, and the retentate was examined for protein by optical density at 280 nm (using an extinction coefficient of 5.5

for a 0.1% solution). HBsAg was assayed by Ausria II (Abbott Laboratories, North Chicago, Ill.), using a reference standard for comparison. In addition, the retentate was subjected to polyacrylamide gel electrophoresis and revealed the presence of only two low-molecular-weight components (P25 and GP30).

The inoculation of humans with this polypeptide vaccine was approved by the Baylor Human Investigations Committee. Six individuals who had previously been vaccinated with the NIH intact HBsAg bulk material in 1979 or 1980 [under a contract (AI92609) from the National Institute of Allergy and Infectious Diseases] and who had responded with the development of anti-HBs were given booster inoculations with the polypeptide vaccine when the anti-HBs concentration fell to nondetectable levels or was <10 mIU/ml. The vaccine was administered by Dr. F. B. Hollinger, who also participated in this trial. Antibody levels were measured at 1, 2, 4, 8, and 12 weeks postinoculation. No side effects were noted, except for occasional mild discomfort at the site of injection. This response was similar to that observed in previous NIH trials with an intact HBsAg particle vaccine.

2. Preparation of HBsAg/adw Polypeptide Vaccine

(a) General Comments: All glassware was siliconized by submersion into a 1% silicone solution prepared by adding 1 ml of Prosil-28 to 99 ml of sterile water and drying overnight at 25°C. Glassware was sterilized before use by moist heat at 120°C for 30 min or by dry heat at 345°F for 15 hr. All buffers (Table 1) were filter-sterilized using sterile 0.22-μm Twin-90 Millipore filter units. After sterilization the buffers were tested for endotoxins, anaerobic and aerobic bacteria, fungi, and mycoplasma. All tubing, columns, pH probes, etc. were autoclaved at 120°C for 30 min or were sterilized by ethylene oxide gas. All steps were carried out in a Baker Class II, Type B, Sterigard hood using sterile gloves and sterile drapes. Finally, all reagents were prepared in pyrogen-free sterile water for irrigation, USP (Travenol).

(b) Disruption of the NIH HBsAg Vaccine, Subtype adw (Lot Cs2R1Cs1-XM-50, 1/13/82): Approximately 10 mg of HBsAg in 68 ml of phosphate-buffered saline (PBS), pH 7.4, was brought to 80 ml with buffer F. One ml was removed and stored at -70°C. To the remainder was added 20 ml of buffer A (100 μg/ml) at a rate of 2 ml/min. After mixing, the sample was placed in a 250-ml Erlenmeyer flask with a stirring bar. The pH was adjusted to 7.2 ± 0.1 with 0.1 N NaOH (pH probe was decontaminated for 1 min in 0.1 N HCl, then rinsed 4 times with sterile, pyrogen-free H_2O prior to use). After sealing the flask with a sterile cap, the sample was placed in a Precision water bath and incubated at $37 \pm 0.5°C$ for 24 ± 2 hr.

(c) Adsorption and Elution of the Polypeptide Complex (P25 + GP30 Complex) by Affinity Chromatography: To prepare the lentil-lectin-Sepharose 4B column containing the polypeptide complex, approximately 10 gm of gel (25 ml) was washed 5 times with buffer B as follows. After settling for 15 min at $23 \pm 3°C$, 15 ml of the supernate was removed from the gel. This volume was replaced with 15 ml of buffer B. The gel was placed on a shaker for 10 min, removed, and allowed to settle for 15 min at $23 \pm 3°C$. This sequence was repeated four times. The washed gel in buffer B was added directly to the disrupted HBsAg, and the mixture was incubated at $23 \pm 3°C$ for 60 min while being continuously shaken.

To a 1 x 30 cm chromatography column, held in place by extension clamps in a base support, was added the lentil-lectin-Sepharose 4B slurry using a 10-ml siliconized glass pipette. As the column was packing, the eluate was collected in a sterile, siliconized 250-ml Erlenmeyer flask until the fluid level was 2 mm above the top of the packed gel. The

eluate was stored at 4-8°C until the final product was analyzed. After packing, the column was washed with 150 ml of buffer B or until the OD_{280} reached a stable baseline tracing. Fractions were collected using a Gilson fraction collector and were monitored at OD_{280} with an ISCO Model UA-6. The tubing and flow-through cell were sterilized by ethylene oxide gas.

(d) Elution of HBsAg Polypeptide Complex (P25 + GP30) from the Column: Before adding the eluting buffer (buffer C), buffer B was allowed to penetrate to the surface of the gel; 5 ml of eluting buffer C then was added to the column. The top of the column was connected via sterile tubing to the barrel of a 60-ml syringe, with the plunger replaced by a sterile cotton plug. The syringe barrel was filled with buffer C as required. The eluate was monitored at OD_{280}, and 2.5-ml fractions were collected into siliconized glass tubes (13 x 100 mm) using a Gilson fraction collector. Fractions containing up to 75% of the protein activity, as monitored by absorbancy at 280 nm, were pooled and transferred into a 50-ml siliconized container. The pool was assayed for HBsAg at a dilution of 1:1000 to verify its presence. This value was compared with that of the original effluent and the original sample.

The pooled fractions containing the HBsAg polypeptides were filter sterilized using a Millex-GV 25-nm, 0.22-μm filter that had been prewashed with 1.0 ml of buffer C. The sample was collected into a 125-ml siliconized beaker.

(e) Microsolute Exchange and Macrosolute Concentration of HBsAg Polypeptide Sample Using an Amicon Ultrafiltration System: An assembled Amicon Model No. 52 with YM10 membrane was sterilized by ethylene oxide gas (<12% gas). At least 2 hr prior to the addition of the sample, 50-75 ml of sterile water was passed through the stirred cell with YM10 membrane in place at a nitrogen (N_2) pressure of 25 ± 5 psi. The nitrogen was sterile-filtered using an in-line Millex-FG filter, 0.2-μm pore size, which was inserted between the nitrogen tank and the stirred cell. Stirring speed was moderate, e.g., the vortex was no greater than one-fourth the height of the starting volume. After removal of glycerin and sodium azide from the YM10 membrane, the sample was added to the filtration cell. The sample volume was reduced to approximately 20 ± 2 ml. Next, buffer D was exchanged for buffer C by adding 6 volumes (180 ml) of buffer D to the Model 52 system by filling the Model 52 stirred cell and allowing the volume to decrease to 20 ml before adding more buffer D until the total volume of buffer D had been expended.

The sample was concentrated to 5.0 ± 1.0 ml at a nitrogen pressure of 25 ± 5 psi using a moderate stirring speed as described above. Before removing the retentate, the pressure was released slowly over 1 min; the stirrer was allowed to continue at atmospheric pressure for an additional 15 min. The sample was withdrawn using a 10-ml siliconized pipette and was placed in a 4-dram, sterile, siliconized vial. Up to 2 ml of buffer D was used to rinse the YM10 membrane. The fluid was allowed to stir for an additional 15 min before it was removed and pooled with the initial sample. Until use, the sample was stored in a refrigerator at 2-8°C.

(f) Micelle Formation of Polypeptide Complex (P25 + GP30) in a Preformed Linear Sucrose Gradient: Beckman SW41 buckets, caps, nitrocellulose tubes (1" x 3.5"), bucket holder rack, and gradient tube holder were gas-sterilized in ethylene oxide at The Methodist Hospital. A 50% (wt/wt) sucrose solution was prepared by adding 500 gm of buffer E to 500 gm of sucrose in a 1-liter beaker containing a 1.5" magnetic stirring bar. Solubilization was allowed to occur for 2-3 hr at 23 ± 3°C with stirring. The refractive index was obtained using a refractometer and was adjusted to 1.420 ± 0.002 with sucrose or buffer E if the value was above or below this range. The sucrose solution was filter-sterilized using a 0.22-μm Twin-90 Millipore filter. Concentrations of sucrose were prepared in 25-ml borosilicate Erlenmeyer flasks as follows.

Concentration of sucrose	Volume (ml) of	
	50% sucrose	Buffer E
50%	25	0
40%	20	5
30%	15	10
20%	10	15

To each of six SW41 nitrocellulose tubes was added 2.5 ml of 50%, 40%, 30% and 20% sucrose solutions in that order. The sucrose gradients were placed in the SW41 buckets, capped, and allowed to equilibrate to form a linear gradient at 2-8°C for 15 ± 3 hr.

From 1.5 to 2.0 ml of the concentrate described above was carefully layered over a sucrose gradient. Six gradients were prepared based on the final volume of concentrate. Additional buffer E was added to raise the liquid meniscus to 0.25" from the top of the tube. The buckets were tightly capped and placed equidistant to each other on the SW41 rotor, which had been precooled to 2-8°C. The rotor and buckets were placed into a precooled (0-4°C) Beckman L3-40 ultracentrifuge chamber, and the chamber was evacuated. At that point the rotor speed was set at 36,000 rpm, and the centrifuge was activated. The temperature control was set to maintain the rotor temperature between 2 and 6°C. Centrifugation was continued for 24 hr, after which the centrifuge was turned off and the rotor was allowed to come to a halt without braking. After releasing the vacuum, the rotor with buckets was removed and taken to the hood where the buckets were placed in the holder rack. After removing the cap, each tube was removed using a sterile hemostat and was placed in a sterile gradient tube holder. Visible bands were recorded diagrammatically.

Sucrose was removed so that the meniscus lay 15-20 mm above the major visible band. Then the tube was side-punctured 3-5 mm below the band with a 20-gauge, regular-beveled, 1" needle that was attached to a sterile Glaspak 10-cc syringe, and the major band was removed. The sample was placed in a 25-ml, sterile, siliconized Erlenmeyer flask. From 0.5 to 1.0 ml of the pooled sample was transferred to a 1-dram, sterile, siliconized vial for biophysical and biochemical characterization (Lowry protein, poly-acrylamide gel electrophoresis, electron microscopy, and HBsAg activity by radioimmuno-assay).

(g) Preparation of Alum-Adsorbed HBsAg Polypeptide Micelles: To prepare a 0.2 M solution of $AlK(SO_4)_2 \cdot 12$ H_2O (mol. wt. = 474.39), sufficient pyrogen-free water was added to 23.72 gm to make 250 ml. The solution was filter sterilized using a 0.22-μm Millipore filter. This preparation contained 5.4 g Al^{+++} per liter, or 5.4 mg/ml.

A Lowry protein determination was performed on the micelle preparation obtained from the sucrose gradient, using hemoglobin and albumin as standards. 16 ml of solution, at a concentration of 180 μg/ml, yielded 2880 μg of polypeptide micelle that was available for adsorption. The amount of aluminum salt needed to adsorb the micelle vaccine in a total volume of 50 ml was as follows:

2880 μg of micelle protein x 0.02 mg Al^{+++}/μg of micelle protein = 57.6 mg Al^{+++}.

57.6 mg Al^{+++} ÷ 5.4 mg Al^{+++}/ml hydrated salt = 10.7 ml hydrated salt.

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50 ml − (10.7 ml hydrated salt + 16.0 ml micelle) = 23.3 ml of buffer F, which was added while stirring (vortex did not exceed 1/8th the height of the fluid in the 25-ml flask). This solution was clear.

The solution was adjusted to pH 6.0 ± 0.2 with 1.0 N NaOH. After this, the solution became opalescent. Adsorption continued at 23-27°C for 2 hr with continuous stirring, monitoring the pH at all times (the pH tends to fall). The flask was closed with a cap, covered with Steri-drape, and centrifuged at 2000 rpm for 10 min. The supernatant was removed by decanting and saved for HBsAg determination. To the adsorbed preparation was added 50 ml of buffer F. The sample was mixed by inversion and centrifuged as before. The supernatant was removed and saved. The vaccine preparation was suspended in buffer F to a calculated concentration of 40 µg of alum-adsorbed micelle protein/ml of suspension: (2880 µg of micelle protein ÷ 40 µg/ml = 72 ml of final PBS suspension in buffer F).

An alum diluent was prepared from the original hydrated salt. To 100 ml was added 1 N NaOH until the pH reached 6.0 ± 0.2 and the solution became opalescent. The alum precipitate was stirred for 2 hr at 23-27°C, pelleted as described above, and reconstituted to contain 0.8 mg of Al^{+++}/ml.

Two 0.5-ml aliquots of the alum suspension were removed, placed in microdialyzing tubes (no. 204; Pope Scientific, Inc., Menomonee Falls, Wisc.), and dialyzed with constant agitation in a 1-liter multiple-dialyzer unit (Model 10) against 3% sodium citrate for 4 hr at 24-26°C. After the solution cleared and the aluminum was completely dissolved, the sample was dialyzed overnight against PBS, pH 7.2 (buffer G), at 4°C with one volume change (1 liter), and the retentate was tested for HBsAg by radioimmunoassay and for viral particles by electron microscopy. The sample was diluted and compared with a standard reference reagent at 10 ng/ml to determine the actual final concentration. In addition, the protein concentration was evaluated by the Lowry method and by spectrophotometry at OD_{280} using an extinction coefficient of 5.5 for a 0.1% solution. Based on these determinations, a final concentration of 38 µg/ml was assigned to the original lot of vaccine.

(h) Distribution of Alum-Adsorbed Preparation into 1.5-ml Vials and Packaging: The 40 µg/ml alum-adsorbed vaccine was placed in a 125-ml sterile, siliconized flask containing a magnetic stirrer. A portion was removed and diluted 1:5 with the alum diluent to prepare a second lot of vaccine containing 8 µg/ml HBsAg polypeptides. Thimerosal was added to a final concentration of 1:20,000. Each lot of vaccine was continuously stirred at a speed of less than 100 rpm. The vaccine was dispensed in 0.7-ml aliquots into 1.5-ml Wheaton "200" glass vials using a 1.0-ml Cornwall automatic pipetter. The vials had a Teflon-lined cap with a septum opening. Thimerosal was added to the alum diluent at a final concentration of 1:20,000. The alum diluent was dispensed in 0.8-ml aliquots into 1.5-ml Wheaton "200" glass vials with Teflon-lined caps as described above.

(i) Sterility Testing of Vaccine and Alum Diluent: Two to four final containers from each lot, before and after addition of thimerosal, were tested for endotoxin, aerobic and anaerobic bacteria, fungi, and mycoplasma by approved techniques. All were negative after 3 weeks.

3. Clinical Trials in Humans

Fifty-one (33 male, 18 female) nonpregnant young adults, health-care students, or professionals were enrolled in the vaccine portion of the study. All were negative for

seromarkers of HBV [anti-HBS, <1.2 sample-to-negative ratio (S/N); anti-HBc, <25% inhibition; HBsAg, <1.0 S/N as tested by the most sensitive methods available (Abbott Laboratories, North Chicago, I'l.)]. Alanine aminotransferase (ALT) and aspartate amino transferase (AST) levels were <30 mIU/ml in all cases, as tested by the Beckman System TR Analyzer. The mean age was 25.7 (range = 21-44 years). Forty of the enrollees were paired by weight (within 11 lbs.), and each member of the pair was randomly assigned to one of two dosage groups (40 or 8 μg/ml). Subsequently, 11 additional young adults were matched by weight with 11 of the original pairs, and each received 1.6 μg of HBsAg/adw polypeptide. The polypeptide micelle vaccine was given intramuscularly in the deltoid region at 0, 1 and 6 months, and blood samples were obtained at 0, 2, 4 and 12 weeks and at 6, 7, 9 and 12 months. All blood samples were processed within 24 hr and were tested for ALT levels and for anti-HBs [expressed in mIU/ml; method of Hollinger et al. (1)]. Anti-HBs levels were considered to be positive when a level of 0.7 mIU/ml or greater was reached. Oral temperatures of the participants were obtained before and 30 min after receiving the vaccine. The volunteers were instructed to take and record their temperatures 4, 8 and 12 hr postinoculation. In addition, any local or systemic reactions were to be recorded.

All 51 participants received three doses of the polypeptide vaccine and had blood samples drawn at the appropriate times. Slightly less than half of the participants experienced pain, erythema or edema at the site of injection (43% after the first inoculation, 45% after the second, and 41% after the third). Systemic symptoms were recorded in 18% of the participants after the first vaccination, in 6% after the second, and in 4% after the third. The most common complaints were fatigue (21% of those reporting symptoms), myalgia other than at the site of injection (21%), and headache or itching (14%). The changes in oral temperature recorded at 30 min averaged less than a third of a degree Fahrenheit, with a range from -0.4 to +1.6°F. There were no differences noted between delta temperatures recorded after any of the three vaccinations. Five volunteers (9.6%) had an elevated ALT level during the course of the study. Four of these elevations occurred on one occasion only (two at 2 weeks, one at 6 months, and one at 7 months after the first inoculation). One participant had engaged in excessive exercise shortly before the phlebotomy and had an elevated creatine phosphokinase along with the abnormal ALT level. Another had been on a new antibiotic, alpalcillin, prior to the finding of the abnormal ALT level. Two others reported nothing in their histories that would account for the elevated enzyme levels. The fifth participant had abnormal liver enzyme levels starting 1 month after the first vaccination, and this abnormality continued intermittently throughout the study; his enzymes returned to within normal limits several times during this period. The patient had a history of excessive alcohol intake but was stated to be abstinent 3 months after the first inoculation. The etiology of these abnormal ALT and AST levels is unknown.

Seroconversion rates by dosage and time are shown in Table 2. The group receiving 40 μg of the polypeptide vaccine had significantly higher seroconversion rates versus those receiving 8 or 1.6 μg/ml after only one inoculation. Two months after the second vaccination, only the 1.6 μg/ml vaccine group had significantly lower seroconversion rates. All differences among the dosage groups disappeared by 6 months, and all volunteers seroconverted by 7 months after the initial inoculation.

Geometric mean (GM) anti-HBs levels are shown in Table 3. The peak GM antibody levels occurred in all three groups at 7 months (1.6 μg/ml group, 120 mIU/ml; 8 μg/ml group, 774 mIU/ml; 40 μg/ml group, 9365 mIU/ml). The group receiving 40 μg/ml had significantly higher GM antibody levels than the groups receiving lower dosages at all time points tested. In addition, after the second dose of vaccine the group receiving 8 μg/ml produced significantly higher levels of antibody than the group receiving 1.6 μg/ml.

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Seven months after the first inoculation, 100% of the volunteers receiving the 40 µg/ml polypeptide vaccine had anti-HBs levels >500 mIU/ml, whereas 65% of those receiving 8 µg/ml and 36% of those receiving 1.6 µg/ml succeeded in attaining these levels.

A multiple linear regression of the dose administered (indicator variable), sex, weight, and age of the volunteer on the antibody level reached at 7 months after the initial vaccination was performed. Although the dose showed a high correlation with the peak antibody response, there was no relationship found between anti-HBs levels produced and weight, sex or age.

The immunogenicity record of the polypeptide vaccine is impressive. As shown in Table 2, seroconversion rates with the 40 µg/ml polypeptide vaccine were similar to those observed in a previous study with the starting material for the polypeptide vaccine, the NIH/40 intact particle vaccine. More importantly, the group that received the polypeptide vaccine had GM anti-HBs levels equivalent to or greater than the GM level of those who had received the NIH/40 vaccine despite the fact that the antigenic content of the polypeptide vaccine (as measured by radioimmunoassay) was less than 2% that of the intact particle vaccine. The 40 µg/ml polypeptide vaccine also produced higher seroconversion rates and higher GM anti-HBs levels than those obtained in a previous vaccination study using 20 µg of HEPTAVAX-B (2).

In addition to the excellent peak antibody response generated by the polypeptide vaccine, the production of anti-HBs in vaccinated individuals was very rapid after the initial immunization. This observation has ramifications for postexposure prophylactic vaccination and indicates that the polypeptide vaccine would be a good candidate for such usage.

It is unclear why no association was found with the polypeptide vaccine between peak anti-HBs level obtained and weight of the vaccinee. This phenomenon has been found in all other studies conducted by our group in those persons under the age of 30. More than 90% of the participants in this polypeptide vaccine study were <30 years of age (mean = 24.7 years), so age was not a factor. The difference between results obtained with this polypeptide vaccine and the intact particle vaccine may lie in differences in conformation. The polypeptide vaccine is in a micellar form, and this may greatly facilitate processing by the immune system, thus over-riding any effects that weight might play. Indeed, the magnitude of the anti-HBs levels obtained in volunteers vaccinated with this polypeptide vaccine lends credence to this theory. If this is the case, this characteristic might be an important one when vaccinating obese individuals.

Our findings with this polypeptide vaccine, which consists of P25 + GP30 HBsAg in micellar form, show that it provides excellent immunogenicity in volunteers with little or no reactogenicity. The rapid immune response generated makes it an excellent candidate in cases where postexposure prophylaxis is needed. In addition, the purity of the polypeptides administered eliminates concerns about host-derived components that may be part of the HBsAg complex and, therefore, part of the intact particle vaccine.

B. Epitopes Associated with HBsAg Synthetic Peptide 1

Earlier studies from our laboratory utilized two cyclic synthetic peptides that were analogous to amino acid residues 122-137 (peptide 1) and 117-137 (peptide 2) of the 25,000 molecular weight polypeptide from HBsAg, subtype ayw. Initial investigations revealed that both peptides were immunogenic in mice after a single injection without conjugation to a protein carrier. However, a poor anamnestic antibody response occurred after a second injection of the peptide. When purified tetanus toxoid was covalently linked to

peptide 1, a vigorous antibody response resulted in mice, similar to that observed with intact 22-nm HBsAg particles. The antibody response was similar regardless of the HBsAg subtype used in the assay, indicating that the major response was to the group a determinant. Recent efforts were directed at defining the epitopes associated with these cyclic peptides; peptide 1 was chosen for these ongoing studies.

Because the cross-reacting group antigenic determinant a of HBsAg is of prime importance in conferring immunity, a synthetic peptide suitable for use as an HBV vaccine candidate should contain the a epitope. To determine which HBsAg antigenic determinants are present on synthetic peptide 1 (SP1), we assessed the ability of the peptide to react with a panel of anti-HBs monoclonal antibodies of known specificity, produced and characterized in our laboratory (3). Cyclic SP1 reacted with 5 of 13 anti-a monoclonal antibodies (4), which indicated that SP1 contains an a epitope and also that the a specificity associated with HBsAg has one or more antigenic determinants, unrelated to that expressed on SP1. After reduction and alkylation of cyclic SP1, the resulting linear peptide no longer reacted with the 5 anti-a monoclonal antibodies. This demonstrated that the disulfide bond is critical in conferring the a specificity and that the a epitope present on cyclic SP1 is conformation-dependent. SP1 also reacted with 3 of 3 anti-y monoclonal antibodies. Reduction of the intrachain disulfide bond and alkylation of free thiol groups did not affect this reactivity, indicating that SP1 contains a sequential y epitope. SP1 did not react with 1 monoclonal antibody with anti-w specificity. The presence of a y epitope on SP1 was not unexpected. Two differences in the amino acid residues of P25 subtype ayw as compared to subtype adw have been reported in the 122-137 region (5). The ayw contains threonine and tyrosine, whereas adw contains asparganine and phenylalanine in positions 131 and 134, respectively. These amino acid substitutions were thought to reflect the subtype y and d activities, as confirmed by our detection of an epitope with y specificity on SP1 (amino acid residues 122-137).

C. Common Idiotype Associated with Human Anti-HBs

The impetus to study idiotypes associated with anti-HBs came from the knowledge that anti-HBs, but not anti-HBc, was protective against HBV infection. We initially generated four rabbit anti-idiotype antisera against affinity purified anti-HBs from two individuals. Each of the four anti-idiotype reagents detected a common anti-HBs idiotype (6); and a single anti-idiotype antiserum was selected for further study. The common human idiotype was detected in purified anti-HBs from three individuals and also in anti-HBs-positive sera obtained from six hemophilic patients. The ability of both HBsAg and a native HBsAg-derived polypeptide to inhibit the idiotype–anti-idiotype reaction suggested that the anti-HBs idiotype was associated with the antibody-combining site (Table 4) (6). Idiotype determinants were detected because the anti-idiotype antisera did not interact effectively with IgG preparations from: (i) the idiotype donor after removal of anti-HBs; (ii) a pool of human sera negative for anti-HBs; and (iii) an individual with a high level of antibody to herpes simplex virus. In addition, the anti-idiotype could not bind either HBsAg or the native HBsAg-derived polypeptide. These data indicated that we were detecting a common human anti-HBs idiotype that was associated with the antibody-combining site (6).

This common idiotype also was expressed on anti-HBs produced in rabbits, guinea pigs, swine, goats, chimpanzees and BALB/c mice that had been immunized with HBsAg (8). However, chickens successfully immunized with HBsAg failed to express the common idiotype. Adsorption studies confirmed that the interspecies idiotype was associated with anti-HBs. These data indicate that the ability to respond to a distinct HBsAg epitope is highly conserved in mammalian species, but may not be shared by avian species.

Further characterization of the common idiotype revealed that it was induced by the group a determinant, because three HBsAg preparations purified from three pools of human plasma positive for the adw, ayw or adr subtypes inhibited the idiotype–anti-idiotype reaction equally on a weight basis (7). Denatured HBsAg viral polypeptides virtually lost their capacity to inhibit the idiotype–anti-idiotype reaction when compared to the native polypeptides (Table 4). Also, reduction of the disulfide bonds and alkylation of free thiol groups destroyed the ability of the native HBsAg-derived polypeptide to inhibit the idiotype–anti-idiotype reaction. These data all suggested that the common anti-HBs idiotype was directed against a conformation-dependent, group-specific a epitope.

We then tested the ability of cyclic SP1 to inhibit the common idiotype–anti-idiotype reaction. On a molar basis this peptide was 10^3-fold less efficient than intact HBsAg in inhibiting the idiotype–anti-idiotype reaction (Table 4) (9). The inability of SP1 to compete equally on a molar basis with HBsAg indicated that it does not represent the complete a determinant and suggested that other amino acid sequences also are important in defining the complete a epitope. The inhibition of the idiotype–anti-idiotype reaction by cyclic SP1 suggested that this sequence is related to antigenic determinants responsible for eliciting a population of human anti-HBs expressing a common idiotype. The importance of conformation again was demonstrated by reducing the disulfide bond in SP1 and alkylating the free thiol groups, which destroyed the ability of SP1 to inhibit the idiotype–anti-idiotype reaction (Table 4) (9).

D. Enhancement of the Anti-HBs Immune Response by Priming with Anti-Idiotype Antibodies

We studied the modulating effects, prior to antigenic challenge, of in vivo administration of anti-idiotype reagents that recognize the common anti-HBs idiotype. Mice injected with alum-precipitated anti-idiotype produced higher anti-HBs titers when compared to mice receiving soluble anti-idiotype before HBsAg stimulation (Table 5) (10). These data suggested that alum precipitation produced a more immunogenic form of the anti-idiotype for enhancing the anti-HBs response. It was noteworthy that primarily IgM anti-HBs was detected by an IgM type-specific radioimmunoassay in the group of mice receiving the soluble anti-idiotype preparation (Table 5), similar to our previous observation that the number of direct IgM anti-HBs plaque-forming units was enhanced by prior injection of soluble anti-idiotype.

The optimal time interval for enhancing the anti-HBs response was determined to be 14 days between primary injection of alum-precipitated anti-idiotype and subsequent inoculation with HBsAg. In addition, the optimal anti-idiotype dose was determined to be 50 μg for enhancement of the anti-HBs response.

The effects of in vivo administration of anti-idiotype antibodies prior to a single injection of cyclic SP1 also were studied. Mice treated with anti-idiotype antibodies prior to injection of cyclic SP1 generated a higher mean anti-HBs titer when compared to mice injected with control antibodies (38.6 as opposed to 4; Table 6) (11). In confirmation of our previous work, mice treated with anti-idiotype antibodies prior to HBsAg produced a higher mean anti-HBs titer when compared with mice receiving pre-IgG prior to HBsAg. It was noteworthy that mice given anti-idiotype antibodies and cyclic SP1 had comparable anti-HBs titers with mice receiving pre-IgG and complete HBsAg (38.6 compared to 34.0). These data indicated that anti-idiotype antibodies administered in conjunction with cyclic SP1 can induce anti-HBs titers comparable to a single injection of whole HBsAg particles.

E. Injection of Anti-Idiotype Antibodies Alone Induces Anti-HBs

As noted above, injection of anti-idiotype antibodies without HBsAg injection produced IgG anti-HBs plaque-forming cells. These data indicated that anti-idiotype antibodies alone can induce an anti-HBs response. Injection of anti-idiotype antibodies alone into mice produced a significant anti-HBs response when compared to mice given a similar injection of control antibodies (Table 7) (12). This anti-idiotype anti-HBs expressed the interspecies idiotype, as inhibition values obtained with a group of 6 sera ranged from 27 to 54%. Conversely, less than 11% inhibition of the idiotype–anti-idiotype reaction was obtained with the non-anti-HBs-containing sera from the 6 mice injected with control antibodies. The specificity of the anti-HBs produced by anti-idiotype injection was determined by inhibition of binding to HBsAg subtype ayw by HBsAg subtypes adw, ayw and adr. Each of the 6 mouse antisera were inhibited from binding to HBsAg/ayw by the 3 HBsAg subtypes, indicating that the anti-idiotype anti-HBs was directed to the a determinant. Therefore, anti-idiotype antibodies can induce anti-HBs that recognizes the a determinant of HBsAg, which has been shown to induce protective immunity to hepatitis B virus (HBV) in humans. In addition, this anti-HBs expresses an idiotype that is shared by anti-HBs produced in humans naturally infected with HBV.

F. Detection of an IgM Anti-Idiotype Directed against Anti-HBs in Hepatitis B Patients

An IgM-specific anti-(anti-HBs) antibody was detected by radioimmunoassay using anti-IgM-coated beads and ^{125}I-labeled anti-HBs (13). This anti-idiotype was found only in the sera of HBV-infected patients, both acute and chronic. Not all HBsAg-positive patients exhibited this reaction, however, and activity was correlated with the presence of HBeAg (Table 8). Approximately 93% of the sera that contained anti-idiotype activity also contained HBeAg. Conversely, 70% of the sera positive for HBeAg reacted in the IgM assay. No correlation was observed between the presence of anti-idiotype and rheumatoid factor or elevated SGPT levels.

Two approaches were used to determine whether the reactive moiety was an IgM anti-(anti-HBs) as postulated or an IgM anti-HBs/HBsAg complex. It was shown that chicken anti-HBs serum, which does not share the common idiotype of human and other mammalian anti-HBs, did not block a positive reaction in this radioimmunoassay even though it specifically bound HBsAg (Table 9). It also was demonstrated that treatment with polyethylene glycol, which will precipitate IgM anti-HBs/HBsAg activity, did not precipitate the reactive moiety in 6 of 7 sera tested (Table 10), lending further evidence to the existence of an IgM anti-idiotype in these patients.

It is suggested that this anti-idiotype directed against anti-HBs may be involved in a defective feedback mechanism, resulting in the suppression of production of anti-HBs and maintenance of the carrier state.

G. Posttransfusion Hepatitis Type A

Hepatitis A virus (HAV) transmission through blood is a rare but potential cause of posttransfusion hepatitis. We documented such a case, supported by laboratory evidence of HAV in the donor blood (14). A 10-year-old girl manifested icteric hepatitis A 31 days after receiving a single unit of packed erythrocytes from a donor who subsequently experienced hepatitis A and died in hepatic failure. HAV antigen was detected in the donor's hepatocytes and in plasma obtained from the original donor unit. The density in cesium chloride of the HAV antigenic activity from the liver and plasma ranged from 1.33 to 1.37 g/cm^3, which is similar to that reported for infectious HAV particles. The

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implicated donor plasma had normal aminotransferase levels and was negative for antibody to HAV. Inoculation of this plasma into a chimpanzee resulted in the development of hepatitis A 23 days later based on the appearance of fecal HAV antigen, hepatitis, and IgM anti-HAV seroconversion. These data clearly documented the presence of HAV in the donor sample that produced posttransfusion hepatitis A.

H. Localization of Hepatitis A Viral Antigen to Specific Subcellular Fractions

HAV antigen has been detected within the cytoplasm of infected cells, but little is known about the precise subcellular localization of HAV once it enters the hepatocyte. Using fresh liver tissue obtained from a chimpanzee experimentally infected with HAV, we employed a gentle procedure to separate various organelles from the liquid phase of the cytoplasm (cytosol) and from the nuclei. Because a large number of enzymes and enzyme systems are localized to the microsomal compartments of the hepatocytes (smooth and rough endoplasmic reticulum) and because enzyme abnormalities frequently accompany HAV infection, we focused our attention on further subfractionating the microsomal compartments in an attempt to localize HAV antigen to a specific site within the hepatocyte.

Most of the antigenic activity (93%) was evenly divided between the cytosol fraction and the microsomal suspension (15). Within the microsomal fraction, more than 75% of the detectable antigen was associated with the smooth endoplasmic reticulum. Less than 4% of the total antigenic activity was localized to the nucleus. These data provided additional evidence that replication of hepatitis A virus occurs primarily within the cytoplasm of the host cell in close association with cellular membranes, consistent with that observed for other members of the genus Enterovirus.

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TABLE 1

Buffers

Buffer	Concen-trates	pH (± 0.1)	Volume (ml)	Chemicals	Weight or volume	
A	5X	7.3	500	10% Triton X-100	50.0	ml
				0.05 M Tris-HCl	3.95	gm
				2.5 M NaCl, USP	73.05	gm
				Pyrogen-free H_2O	400.0	ml
				Titrate to pH 7.3 with 1.0 N NaOH		
				Pyrogen-free H_2O, q.s.[a] to make	500.0	ml
B	1X	7.3	1000	10% $CaCl_2$	1.11	ml
				0.001 M $MnCl_2$	0.198	gm
				1X buffer A, q.s. to make	1000.0	ml
C	1X	7.3	500	0.01 M Trizma, pH 7.3	0.765	gm
				5% α-methyl-D-mannoside	25.0	gm
				2% Triton X-100	10.0	ml
				Pyrogen-free H_2O	400.0	ml
				Titrate to pH 7.3 with 0.1 N NaOH		
				Pyrogen-free H_2O, q.s. to make	500.0	ml
D	1X	7.3	1000	0.01 M Tris-HCl	1.58	gm
				Pyrogen-free H_2O, q.s. to make	1000.0	ml
E	1X	7.3	2000	0.05 M Tris-HCl	15.8	gm
				0.10 M NaCl, USP	11.69	gm
				Pyrogen-free H_2O	1900.0	ml
				Adjust to pH 7.3 with 0.1 N NaOH		
				Pyrogen-free H_2O, q.s. to make	2000.0	ml
F	1X	6.2	1000	0.00775 M $NaH_2PO_4 \cdot H_2O$, USP	1.0694	gm
				0.00225 M $Na_2HPO_4 \cdot 7\ H_2O$, USP	0.6032	gm
				0.15 M NaCl, USP	4.38	gm
				Pyrogen-free H_2O, q.s. to make	1000.0	ml

[a] q.s. = Quantity sufficient.

TABLE 2

Seroconversion Rates (%) by Dosage and Time

Dosage, µg	Time (months)						
	0.5	1	3	6	7	9	12
1.6 (n = 11)	27	36	55	82	100	90	91
8 (n = 20)	35	45	100[a]	100	100	100	100
40 (n = 20)	80[b]	90[b]	100[a]	100	100	100	100
40 (n = 25)[c] (NIH intact particle vaccine)	NT[d]	84	100	100	100	100	100

[a] $p < 0.01$, 40 or 8 µg vs. 1.6 µg polypeptide vaccine.

[b] $p < 0.015$, 40 µg vs. 8 or 1.6 µg polypeptide vaccine.

[c] Data taken from Hollinger et al. (1).

[d] NT = not tested.

TABLE 3

Geometric Mean Anti-HBs Levels (mIU/ml)

Dosage, µg	0.5	1	3	6	7	9	12
				Time (months)			
1.6 (n = 11)	0.5	0.8	2.4	6.5	120	44	21
8 (n = 20)	0.5	1.1	17.0[a]	35.0[a]	774[a]	424[a]	185[a]
40 (n = 20)	4.7[b]	6.8[b]	201.0[b,c]	276.0[b]	9365[b]	3986[b]	1758[b]
40 (n = 25)[d] (NIH intact particle vaccine)	NT[e]	3.7[b]	87.0[b]	192.0[b]	3335[b]	3273[b]	1903[b]

[a] $p < 0.05$, 1.6 µg vs. 8 µg polypeptide vaccine.

[b] $p < 0.05$, 40 µg vs. 8 or 1.6 µg polypeptide vaccine.

[c] $p < 0.05$, 40 µg polypeptide vaccine vs. 40 µg NIH intact particle vaccine.

[d] Data based on Hollinger et al. (1).

[e] NT = not tested.

TABLE 4

Percent Inhibition of the Common Anti-HBs Idiotype
Binding Its Anti-Idiotype Antiserum by Different
Concentrations of Various Inhibitors

Inhibitor	Concentration (μg)	Inhibition[a]
Native HBsAg-derived polypeptide[b]	7.5	63
	3.75	45
	0.75	21
	0.375	18
Native HBsAg-derived polypeptide, reduced and alkylated[c]	7.5	2
	0.75	0
	0.075	0
SDS-denatured P25 and GP30[d]	25.0	9-13
	2.5	2-5
Synthetic peptide 1	250	63
	25	33
	2.5	30
Synthetic peptide 1, reduced and alkylated	25	1
	2.5	0
Human serum albumin	20.0	0
	5.0	0

[a] The inhibition values represent the mean of triplicate determinations. A solid-phase radioimmunoassay was used to determine the percent inhibition of the idiotype–anti-idiotype reaction.

[b] Aggregate of P25-GP30 isolated as a micelle by lentil lectin affinity chromatography of HBsAg solubilized with 2% Triton X-100 and 0.5 M NaCl.

[c] Native HBsAg-derived polypeptide micelles and synthetic peptide 1 were reduced with a 3-fold molar excess of 2-mercaptoethanol and alkylated with a 10 molar excess of iodoacetamide under N_2 atmosphere.

[d] Isolated by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions.

TABLE 5

Comparison of Alum-Precipitated and Soluble Anti-Idiotype for Induction of Anti-HBs[a]

First injection	Second injection	No. of mice	Anti-HBs response (mean ± SEM)[b]	Micro-SPRIA Anti-HBs IgG[c]	Micro-SPRIA Anti-HBs IgM[d]
Anti-idiotype alum. ppt.	HBsAg	4	487.5 ± 315.0	4938	1250
Anti-idiotype soluble	HBsAg	4	72.5 ± 50.0	86	1000
Pre-rabbit IgG alum. ppt.	HBsAg	4	< 5.0[e]	<5.0	<5.0
Pre-rabbit IgG soluble	HBsAg	4	6.25 ± 1.08	30	50

[a] Each group of mice received 40 µg of anti-idiotype or pre-IgG on day 0, followed by 6 µg of HBsAg on day 14, all by the intraperitoneal route. Mice were bled on day 26.

[b] The values are the reciprocal dilution of antisera which gave an endpoint S/N of 2.1 as measured by AUSAB.

[c] The mean value of reciprocal dilution of antisera which gave an endpoint S/N of 2.1 as measured by solid-phase radioimmunoassay (SPRIA) using ^{125}I-labeled goat anti-mouse γ-chain specific antiserum.

[d] The mean value of reciprocal dilution of antisera which gave an endpoint S/N of 2.1 as measured by SPRIA using ^{125}I-labeled rabbit anti-mouse µ-chain specific antiserum.

[e] All mice were negative for anti-HBs at a serum dilution of 1:5.

TABLE 6

Priming the Anti-HBs Response by Prior
Injection of Anti-Idiotype Antibodies[a]

First injection	Second injection	No. of mice	Anti-HBs response (mean ± SEM)[b]
Pre-IgG	peptide 1	6	4.0 ± 1.9
Anti-idiotype	peptide 1	7	38.6 ± 9.6
Pre-IgG	HBsAg	5	34.0 ± 8.7
Anti-idiotype	HBsAg	6	10,416 ± 306

[a] All mice received 50 µg of either alum-precipitated anti-idiotype or control, preimmune rabbit IgG on day 0, followed by 50 µg of peptide 1 or 6 µg of HBsAg on day 14, all by the intraperitoneal route. Serum was obtained on day 30, and the reciprocal of the endpoint dilution which gave an arbitrary positive-to-negative cpm ratio of 2.1 was determined by radioimmunoassay as previously described.

[b] Mean of reciprocal endpoint titer (± standard error of the mean) in the responding animals.

TABLE 7

Anti-HBs Response in Mice Injected with Anti-Idiotype Antibodies[a]

First injection	Second injection	Anti-HBs titer (ayw)	% inhibition			% inhibition of idiotype–anti-idiotype reaction
			adw	ayw	adr	
Anti-idiotype	Anti-idiotype	750[b]	85	85	70	38
Anti-idiotype	Anti-idiotype	1000	88	86	78	46
Anti-idiotype	Anti-idiotype	1000	96	92	84	51
Anti-idiotype	Anti-idiotype	1250	96	96	90	54
Anti-idiotype	Anti-idiotype	250	90	84	76	27
Anti-idiotype	Anti-idiotype	750	80	86	71	34
Pre-IgG[c]	Pre-IgG	< 5	ND[d]	ND	ND	0-11

[a] Anti-HBs response expressed as the reciprocal dilution of antiserum which bound HBsAg, subtype ayw, and gave an arbitrary positive (S) to negative (N) counts per minute ratio of 2.1. In addition, the percentage inhibition of binding a constant dilution of mouse antiserum to HBsAg, subtype ayw, by 5 µg of HBsAg subtypes ayw, adw and adr was determined. Each group of 6 mice was given 50 µg of alum-precipitated anti-idiotype or pre-IgG on day 0, followed by a similar injection on day 14, all by the intraperitoneal route. Serum was obtained on day 26, and the endpoint anti-HBs titer and S/N ratios for binding the three serotypes of HBsAg were determined. The ability of these mouse sera to inhibit the human idiotype–anti-idiotype reaction was examined at a 1:10 dilution.

[b] Statistical method using two-tailed Student's t test was based on the $\log_{10}$ arithmetic mean titer.

[c] All 6 mouse antisera were negative at the dilution tested.

[d] ND = Not determined.

TABLE 8

HBeAg/Anti-HBe Status of HBsAg-Positive Sera Tested for
IgM Antiglobulin (Anti-Idiotype) Activity Against Anti-HBs

HBeAg/anti-HBe status of HBsAg-positive sera	No.	% positive for anti-(anti-HBs)
HBeAg positive only	87	70.1
Anti-HBe positive only	44	9.1
Both positive	2	50.0
Neither positive	7	14.3
Total	140	47.9

TABLE 9

Results of Inhibition Assay Using Species-Specific Anti-HBs Reagents

Blocking reagent	Beads coated with anti-IgM[a]		Beads coated with HBsAg[b]	
	S/N	% inhibition	S/N	% inhibition
Normal goat serum	7.7	-	39.3	-
Goat anti-HBs	2.2	71	5.5	86
Normal guinea pig serum	6.6	-	51.6	-
Guinea pig anti-HBs	2.1	68	22.7	56
Normal chicken serum	6.4	-	43.5	-
Chicken no. 1 anti-HBs	5.0	22	10.0	77
Chicken no. 2 anti-HBs	5.4	16	12.2	72

[a] A previously determined positive specimen was added to beads coated with anti-human IgM followed sequentially by the addition of species-specific anti-HBs and ^{125}I-labeled human anti-HBs.

[b] Species-specific anti-HBs reagents were added to beads coated with HBsAg following which ^{125}I-labeled human anti-HBs was added.

TABLE 10

Distribution of Specific Activity in Samples
after Polyethylene Glycol Precipitation

Type of sample	% activity found in	
	Supernatant	Pellet
^{125}I-labeled HBsAg	100	0
IgM-specific anti-HBs	92	8
IgM-specific anti-HBs/^{125}I-HBsAg complex	16	84
Human serum A[a]	100	0
B	100	0
C	100	0
D	100	0
E	88	12
F	70	30
G	0	100

[a] A to G are sera that were positive in the IgM assay.

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